Age, caste, and behavior determine the replicative activity of intestinal stem cells in honeybees (*Apis mellifera* L.)

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Ward, K. N., Coleman, J. Clittin, K., Fahrbach, S., Rueppell O. (2008) Age, caste, and behavior determine the replicative activity of intestinal stem cells in honeybees (*Apis mellifera* L.). Experimental Gerontology, 43: 530-537.

Abstract:

Honeybees (*Apis mellifera* L.) display a pronounced natural aging plasticity. The differences in aging rates between the alternative phenotypes and behavioral classes could reflect differences in protection against damage or in the ability to repair vulnerable tissues. As in other animals, including humans, the gut is continually exposed to environmental insults and harbors a large population of replicating stem cells that maintain the intestinal epithelium. Through studies of the major internal organs using incorporation and immunodetection of the mitotic marker bromo-deoxyuridine, the intestine was determined to be the main site of tissue renewal in adult honeybees. Proliferative activity of the intestinal stem cells was compared among queens, workers, and males of different ages. Simultaneous attempts to assess intestinal cell loss via apoptosis yielded inconclusive results. The relationship between intestinal cell proliferation and worker life-history was evaluated in greater depth by studying *diutinus* winter workers, reproductive workers, and by decoupling worker behavioral status from chronological age in a single-cohort colony. Intestinal cell proliferation was abundant in all groups and showed an age-related decline in workers, queens, and males. At young ages, workers exhibited relatively more intestinal cell proliferation than did queens and queens more than drones, but the caste and sex differences decreased with age. Cell proliferation did not decrease beyond 6 weeks of age in older queens and in *diutinus* workers. Ovary activation did not correlate with the amount of intestinal stem cell proliferation in workers, although the queenless hive condition was associated with lower overall counts. In the single-cohort colony, nurse bees exhibited more cell proliferation than foragers, regardless of age. The overall results do not support our hypothesis that longer-lived phenotypes exhibit increased somatic repair in the form of higher replicative activity of intestinal stem cells. Instead, the observed proliferation patterns reflect differential demands for digestive activity in the different groups, which result in different requirements for replacement of lost intestinal cells. The maintenance of proliferative capacity for over 1 year suggests that queen intestinal stem cells have a relatively high replicative potential, but further studies are needed to relate honeybee lifespan differences to cellular aging.

Keywords: Aging plasticity; Castes; Cell proliferation; Evolution; Longevity regulation; Midgut; Replicative senescence; Social insects

Article:

1. Introduction

Naturally occurring plasticity in aging and lifespan variation has recently attracted considerable interest because it may lead to the discovery of longevity mechanisms that have not been revealed by classic genetic or pharmacological approaches ([Keller and Genoud, 1997], [Tatar and Yin, 2001], [Gardner et al., 2006] and [Jaureguy and Etges, 2007]). Furthermore, aging plasticity, apparent as intra-specific variation in life expectancy, is the rule rather than the exception: species have not only evolved an average lifespan but also a certain degree of plasticity in response to social or environmental selection ([Carey, 2001] and [Carey et al., 2005]). Alternative developmental pathways leading to polyphenisms present the extremes of this plasticity (West-Eberhard, 2003). The comparative focus on alternative phenotypes shifts the emphasis from the study of single genes to systemic analyses of gene expression networks that are connected to aging differentials at the cellular, organismal, or biodemographic levels of biological organization.

The honeybee, *Apis mellifera* (L.), is an attractive model in which to explore the natural causes and consequences of alternative developmental pathways that lead to a pronounced aging plasticity. Honeybees live in familial societies that each contain one long-lived, reproductive queen and many, short-lived males (drones) and non-reproductive, female workers (Page and Peng, 2001). Queens, drones, and workers differ in anatomy, physiology, behavior, and life-history but develop from the same genotype through alternative developmental pathways. Drones develop from unfertilized, haploid eggs due to the complementary sex determiner locus (Beye et al., 2003). Fertilized eggs usually develop into females and remain bipotent until they commit to queen or worker development during the third larval instar (Winston, 1987). This commitment is based on differential feeding that affects hormone titers (Page and Peng, 2001) and the ILS and TOR signaling-pathways ([Wheeler et al., 2006] and [Patel et al., 2007]). In comparison to worker larvae, queen-destined larvae grow faster and bigger and develop a complete reproductive system.

Queens, drones, and workers fulfill very different societal roles and consequently exhibit divergent life-histories. After successful mating, the young queen inherits her natal hive and initiates egg-laying as her sole subsequent responsibility until death, which may be controlled by her workers (Page and Peng, 2001). Throughout her life, the queen is continuously groomed and fed by workers and can lay approximately 2000 eggs per day (Camazine, 1991). Longevity reports on queens are variable but it is clear that queens can live multiple years, with a possible maximum of up to 8 years (Page and Peng, 2001). Drones are also entirely, yet very differently, specialized for reproduction. After an initial week of maturation, they leave the nest for repeated mating flights until they die. Copulation leads directly to drone death, but most drones die of other causes after a relatively short life of up to 54 days with an average of 30 days (Rueppell et al., 2005).

Workers fulfill all non-reproductive tasks in the colony and show the most variable life-history. They usually go through an age-based division of labor schedule, passing from in-hive nursing, nest-construction, and food processing to foraging outside the hive (Winston, 1987). These behavioral transitions are accompanied by significant physiological changes including glandular activation and degeneration ([Snodgrass, 1956] and [Winston, 1987]). The most distinct transition takes place at the onset of foraging, which is a major life-history determinant in honey bee workers (Rueppell et al., 2007). This transition is only reversed under special circumstances

([Robinson et al., 1992] and [Amdam et al., 2005]). In the absence of a queen and brood, workers can activate their rudimentary ovaries and lay fertile, male eggs (Hoover et al., 2006). Worker life expectancy depends on season and colony status and usually varies in the summer around a mean of 3 weeks, with a maximum around 60 days (Rueppell et al., 2007). It may extend past 6 months in the winter despite physiological activity for hive thermoregulation (Omholt and Amdam, 2004).

The causes and consequences of the plasticity in life expectancy among the different members of honeybee society have only recently begun to be addressed. The relatively high mortality of drones and summer workers appears to be linked to the onset of flight ([Rueppell et al., 2005] and [Rueppell et al., 2007]) but the increased external mortality risk outside the hive is not solely responsible for their short lifespan, at least in not workers (Rueppell et al., 2007). Down-regulation of the important yolk protein vitellogenin has been associated with the transition from hive worker to forager (Amdam et al., 2007). Conversely, elevated titers of vitellogenin may contribute to the longer-lived *diutinus* worker sub-caste that is the typical phenotype of winter workers (Omholt and Amdam, 2004). Vitellogenin has antioxidant functions (Seehuus et al., 2006) and is an important storage and transport protein (Amdam et al., 2004). Furthermore, the long-lived queens maintain higher levels of vitellogenin than drones and workers and are more resistant to paraquat-induced oxidative stress (Corona et al., 2007) and lipid peroxidation (Haddad et al., 2007) than workers. These recent data suggest that cells may accumulate less molecular damage in long-lived individuals, such as queens, than in short-lived ones (e.g. summer workers and drones).

Long life, however, may also be achieved by and be correlated with elevated levels of replacement of damaged or dying somatic cells by stem cells (Rando, 2006). Tissue regenerative potential generally declines with age, leading to failures in tissue homeostasis and function (Buetow, 1985). This phenomenon may be attributed to the depletion of stem and progenitor cells ([Lobachevsky and Radford, 2006] and [Ruzankina and Brown, 2007]), resulting in declining cell proliferation rates. So far, this concept has mainly been explored in vertebrates because other aging models, such as adult insects, have been regarded as largely postmitotic (Finch, 1990). But cell proliferation has been observed in adult insect tissues ([Cayre et al., 1994] and [Corley and Lavine, 2006]) and the insect intestine in particular is characterized by high levels of regenerative stem cell proliferation (Ohlstein and Spradling, 2006). Furthermore, an age-related decline in digestive efficiency in honeybees has been reported (Crailsheim et al., 1992). This makes replicative cell senescence potentially relevant for aging in insects and provides a possible explanation for natural telomere length differences in insects that are related to lifespan differences (Jemielity et al., 2007). The intestine is in many animals a high cell turnover tissue in which age-related decreases in cell proliferation have been reported (Hamilton, 1985). As in other tissues, cell proliferation has to be in a dynamic equilibrium with cell death (Ginaldi et al., 2000), and apoptosis, the predominant form of programmed cell death, has been implicated repeatedly in aging (Muradian and Schachtschabel, 2001).

We systematically screened the major tissues of adult honeybees (*A. mellifera* L.) for evidence of active cell proliferation, with the goal of investigating the potential connection between the patterns of cell proliferation and apoptosis and the natural differences in life expectancy of queen, drone, and worker honeybees. We also investigated the association of intestinal cell

proliferation with the aging plasticity of workers by comparing nursing and foraging workers at young and old ages, studying *diutinus* workers, and measuring intestinal cell proliferation in queenless colonies in which workers had become reproductively active. Finally, we fed the cytostatic drug hydroxyurea to workers and measured their subsequent intestinal cell proliferation and survival. Overall, we did not confirm our central hypothesis of a direct link between the rate of cellular renewal and aging plasticity, but the patterns of intestinal cell proliferation were more consistent with different digestive demands in our experimental groups.

2. Materials and methods

2.1. Cell proliferation assay

To examine cellular proliferation we used a 5-bromo-2′-deoxyuridine (BrdU: Sigma) incorporation assay (Shermoen, 2000). Although BrdU can be incorporated into endo-replicating DNA, the pattern of labeling in our samples, as assessed with an Olympus Fluoview FV5OO/IX81confocal microscope, suggests that, in our samples, the BrdU label indicated DNA replication linked to cell proliferation (Fig. 1).

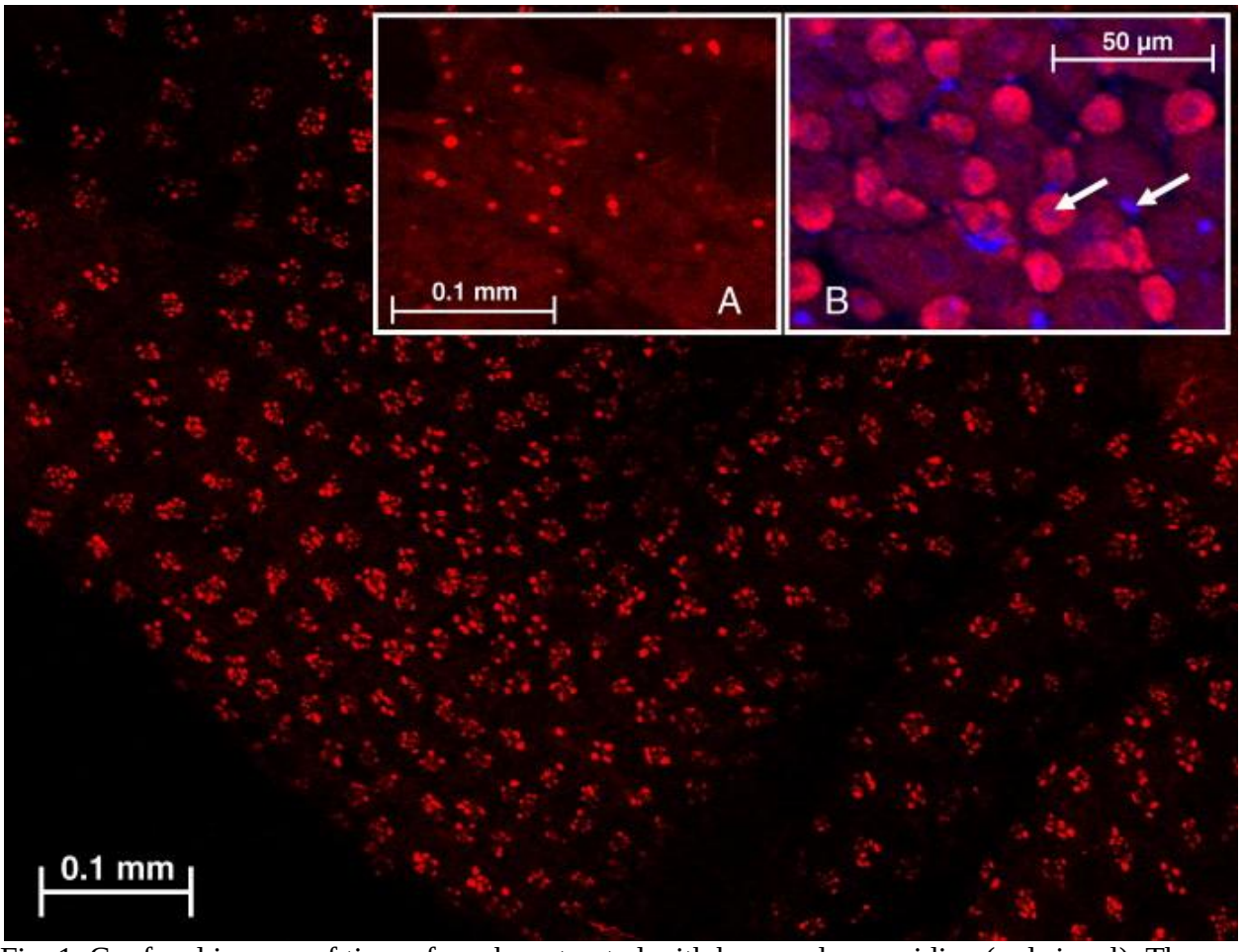

Fig. 1. Confocal images of tissue from bees treated with bromo-deoxyuridine (red signal). The label in the intestine (large picture) was consistent with histological data and a second alternative staining method (Fig. 2). In contrast, the labeling in pericardial tissues (inset A) was sporadic and in the fat body (inset B) the signal from the cytoplasm of a subset of cells may have been due to

autofluorescence (DAPI-stained nuclei (blue) from a labeled and an unlabeled cell are indicated with white arrows).

Honey bees were collected from the colonies, starved for 30 min in an incubator (34 °C, 60% relative humidity), and harnessed in plastic straws with their proboscis accessible. Each bee was then fed 5 µl of a solution containing 5 mg/ml BrdU and 25% sucrose. Bees that did not consume the entire 5 µl were discarded. The BrdU-treated bees were subsequently placed back into the incubator overnight for a total of 16–20 h to allow BrdU to incorporate into replicating DNA. After the BrdU treatment was administered, bees were dissected in bee saline (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, and 10 mM Hepes, pH 6.7). The tissues of interest were fixed in Carnoy's fixative (60 ml of 100% ethanol, 30 ml chloroform, and 10 ml acetic acid) for one hour. Next, the samples were dehydrated in 100% ethanol three times, placed once in a 1:1 mixture of xylene and ethanol, once into 100% xylene, once into a 1:1 mixture of xylene and Paraplast (Fisher Scientific), and once into 100% Paraplast, each step for fifteen minutes. Finally the tissues were placed in fresh Paraplast to harden overnight for 16–20 h. The Paraplast was remelted at 60 °C. The tissues were embedded and sectioned at a thickness of 10 µm. Sections were deparaffinized with three washes of xylene and then rehydrated with a series of ethanol in distilled water mixtures (100%, 100%, 95%, and 70% ethanol) for 5 min each.

After rehydration, the sections were rinsed briefly in distilled water to remove any excess ethanol and then permeabilized with 1× PBS-T (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, and 0.05% Triton X-100, pH 7.4) twice for five minutes. The DNA was denatured in 1× PBS-T containing 2 N HCl for one hour and subsequently washed twice in PBS-T for 5 min each. Tissues were blocked for non-specific binding with PBS-T containing 0.05% normal goat serum (Biomeda) and 0.2 g/l of BSA (Sigma) for 1 h. Slides were incubated with anti-BrdU mouse antibody (Phoenix Flow Systems) diluted 1:500 in PBS-T overnight for 16–20 h at 4 °C. The samples were then washed three times in PBS-T for 10 min each time before incubation with goat anti-mouse peroxidase conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:40 in PBS-T for 2 h. After washing three times with PBS-T and twice with 1× PBS for ten minutes each, tissues were stained with SigmaFast™ diaminobenzidine (Sigma) for 5–10 min until slides were visibly stained. Following the diaminobenzidine incubation, the samples were rinsed three times in distilled water for one minute each and then dehydrated using an ascending series of ethanols for 5 min each. Citrus clearing solvent (VWR) was used three times for 5 min each before slides were mounted with Permount (Fisher). Negative controls included the omission of HCl to maintain DNA structure and therefore inhibit antibody access to incorporated BrdU and omission of the anti-BrdU antibody.

2.2. Apoptosis assay

Initially, the APO-BRDU™ kit (Phoenix Flow Systems) was slightly modified for whole tissue staining from the manufacturer's recommendations but did not yield consistent labeling results. Therefore, we subsequently used the DeadEnd™ Colorimetric TUNEL labeling system (Promega). Tissue sections were generated, deparaffinized, and rehydrated as previously described. The samples were then rinsed briefly in distilled water to remove excess ethanol, washed in 0.85% NaCl and 1× PBS each for 5 min, and fixed in 4% paraformaldehyde in 1×

PBS at pH 7.50 for 15 min. Subsequent treatment followed the manufacturer's recommendations, including negative controls by omitting the rTdT enzyme from the rTdT reaction mix and positive controls by treating tissues with DNase I (Promega). Further positive controls were performed using the midguts of *Drosophila melanogaster* larvae with known apoptosis (Mills et al., 2006). Quantification of labeled apoptotic nuclei was carried out as previously described in the cellular proliferation assay.

2.3. Experiments

Initially, various tissues from honeybee queens, workers, and drones were screened for signs of cell proliferation at different ages. We screened the hypopharyngeal and salivary glands, reproductive organs, brain and abdominal ganglia, the heart and associated tissues, head and abdominal fat body, entire digestive system (crop, midgut, and rectum), malphigian tubules, and the flight muscles.

Once the midgut wall was identified as the only tissue displaying substantial amounts of cell proliferation, a second experiment was performed to compare intestinal cell proliferation and apoptosis rates among the long-lived queens and the shorter-lived workers and drones. For each bee, one cross-section was randomly selected from the center (middle section) of the intestine. The total number of labeled nuclei was counted from that section at 100× magnification with a Nikon Eclipse e200 light microscope. Comparisons were based directly on these counts because no differences in intestinal cell size or gut diameter were evident across the different experimental groups. For all experiments, group means are reported with their 95% confidence intervals (C.I.).

To obtain honey bee workers and drones of known age, empty combs were placed into standard hives to induce egg-laying. Bees were allowed to emerge in an incubator (34 °C, 60% relative humidity), marked with enamel paint (Testors, Rockford, IL) on their thoraces, and re-introduced into their colony. Queens were obtained by grafting first and second instar larvae into artificial queen cells. These cells were introduced to a queenless rearing colony and transferred to the incubator 1–5 days prior to emergence. They were also paint-marked before their individual introduction into small, queenless hives. Cohorts were compared throughout the summer because preliminary studies suggested no temporal effect during the active season. Workers were collected in three different age classes (1–3 days old, 10–15 days old, and 41–51 days old). These age classes correspond to the three main stages of worker life-history: young hive bees that are pre-nurses, mature hive bees that have almost completed their hive tenure, and foragers ([Crailsheim, 1990] and [Winston, 1987]). Queens and drones of similar ages were used for comparison but for queens an additional older group (>1 year) was studied.

To obtain *diutinus* winter workers, we monitored hives repeatedly but brood rearing was never completely abandoned in North Carolina. Finally, we were shipped a colony of wintering bees from Indiana (G. Hunt, Purdue University). These workers were of unknown age, but at least 2 months old. Immediately after arrival, 20 workers were processed for the BrdU-based cell proliferation assay, as described above.

To decouple worker age and behavioral profile, a single-cohort colony (Huang and Robinson, 1992) was set up in an observation hive with newly emerged, individually tagged workers.

Foraging activity was monitored for 2 h per day throughout the experiment and 15 foragers and 15 nurses were collected at 8 and 33 days of age. Workers were collected as foragers when they returned from a foraging trip and if they had previously been recorded as foragers. Individuals were collected as nurses if they had never been recorded foraging and were observed directly engaged in nursing activities during the time of collection.

To assess how reproductive activity directly affects the rate of intestinal cell proliferation, we set up a queenless colony in an observation hive and added 1000 newly emerged, individually tagged workers. Individual egg-laying was recorded daily and between 25 and 31 days of age, workers with and without egg-laying records were collected and immediately subjected to the BrdU-incorporation assay as described above. The state of ovarian activation was also assessed in these workers using a conventional 5-point scale (Pernal and Currie, 2000) to correlate ovary activation and intestinal cell proliferation.

In a final set of experiments, we fed the cytostatic (S-phase inhibitor) drug hydroxyurea (Sigma) to honeybee workers to inhibit intestinal cell proliferation and measure the effects of this treatment on mortality. Workers were allowed to emerge overnight in an incubator (34 °C, 60% relative humidity), color-marked, introduced into a host colony, and collected after 7 days. In the first experiment using hydroxyurea, workers were kept for 48 hours in the incubator (30 °C, 50% relative humidity) with access to *ad libitum* food (queen candy: powdered sugar-in-water paste) containing either 30 mg/g hydroxyurea (treatment) or no drug (control). In a second hydroxyurea experiment, workers were hand-fed with treated or untreated queen candy until saturation. In both studies, workers were briefly anaesthetized with CO_2 after feeding to allow for individual marking with colored number tags (BeeWorks, Canada). A small subgroup of bees was used from both experiments for assessing the treatment effect on intestinal cell proliferation with the BrdU assay described above. The remaining bees were introduced into their colony in an observation hive to monitor their survival. This re-introduction caused high initial mortality. But in the first hydroxyurea study, 20 control and 20 treatment bees were successfully introduced into the colony, while another group of 22 treated and 18 control bees was placed in the colony in a wire mesh cage that allowed contact but prevented any of the introduced workers from leaving the colony. In the second study, 16 treated and 14 untreated bees were freely introduced into the hive. All except two disappeared during the first 12 h, indicating rejection by the host hive. Survival was assessed daily by a detailed census of the hive. Worker death was assumed on the day that an individual was last recorded.

2.4. Statistics

The overall effect on the amount of cell proliferation of age and group (queen, drone, and worker) and their interaction was assessed by two-factorial ANOVA, followed by Dunnett's T3 post hoc tests to assess exact group differences with unequal variances. The same statistical approach was used to assess the effects of age and behavioral group on the amount of cell proliferation in workers. To investigate age-related trends within single groups, linear regressions were performed, followed by ANOVA and Dunnett's T3 post hoc tests. Further group comparisons were also performed by simple ANOVAs. For the correlation of the categorical ovary activation score with the amount of cell proliferation, we used Spearman's non-parametric correlation analysis, and the survival effect of hydroxyurea treatment was assessed by a Mantel-Cox Log rank test.

3. Results

Among all tissues screened for BrdU-incorporation, only the intestine (midgut) provided clear and consistent evidence of DNA synthesis. Confocal scanning microscopy revealed labeled nuclei, mostly in multiples of two, arranged in clusters at the base of the intestinal crypts (Fig. 1). The arrangement and size estimates of the labeled nuclei exclude the possibility of staining due to endoreplication. In addition, sporadic labeling was found in the pericardial tissue (Fig. 1, inset A) and strong labeling was detected in a subpopulation of fat body cells (Fig. 1, inset B). The latter two results, however, could not be confirmed with a non-fluorescent protocol. We also noted that the fluorescence in the fat body was localized to the cytoplasm, rather than to the nucleus. This makes autofluorescence of non-specific labeling a probable explanation of the signal in the fat body. Consequently, the intestine was identified as the only relevant tissue for a comparison between longevity and the balance of cell proliferation and apoptosis. Thin cross-sections of the BrdU-labeled intestines (Fig. 2) proved to be more consistent quantitative measures of the amount of cell proliferation than the confocal images, and all subsequent results are based on the counts of all labeled nuclei per representative cross-section.

Fig. 2. Cross-section of a BrdU-labeled honeybee worker intestine. The replicating cells are apparent as dark brown clusters at the bottom of the intestinal crypts (white arrows).

An overall analysis of the data from the comparison between the three principal honeybee groups (queens, drones, and workers) across their lifespans revealed significant effects of age ($F_{(3,175)} = 38.3$, $p < 0.001$), group ($F_{(2,175)} = 29.6$, $p < 0.001$), and their interaction ($F_{(1,175)} = 13.5$, $p < 0.001$) on the number of labeled cells in the intestine (Fig. 3). Dunnett's T3 post hoc tests suggested that the overall group effect was due to higher counts in workers than in queens

($p = 0.003$) or drones ($p < 0.001$). At young ages (1–2 days), workers had significantly higher counts than queens ($p = 0.001$) and queens had significantly higher counts than drones ($p < 0.001$). At middle age (11–12 days), the only significant group effect was a higher count in workers than in drones ($p = 0.024$), and at the oldest age (41–42 days) no significant group differences were found (Fig. 3).

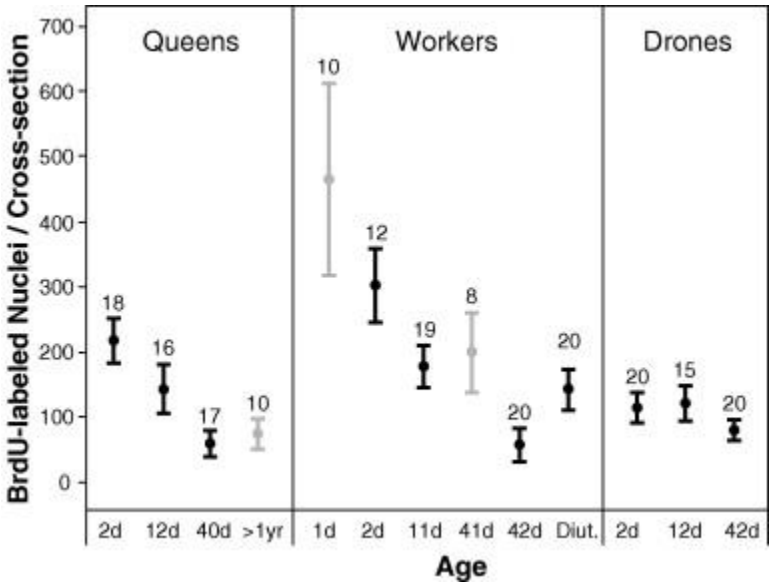

Fig. 3. The amount of intestinal cell proliferation declined with age in honeybee queens, workers and drones. The initial differences between groups also declined with age. Shown are group means and 95% C.I., in black from 2006 and in gray from 2007. "Diut." refers to a group of *diutinus* winter workers that were older than 2 months.

The decline with age occurred in all age groups. Cell proliferation in queens significantly decreased with age from 2 to 40 days ($r^2 = 0.54$, $F_{(1,49)} = 58.6$, $p < 0.001$) but no subsequent decline ($F_{(1,24)} = 1.0$, $p = 0.321$) was apparent when the 40-day-old queens were compared with the >1 year olds (Fig. 3). Post hoc tests revealed that the youngest age class showed significantly more labeling than all other classes ($p_{middle} = 0.021$, $p_{old} < 0.001$, $p_{year} < 0.001$) and the middle age class more than the 40-day-old ($p = 0.002$) and >1-year-old ($p = 0.017$). Similarly, there was a strong age-related decline in worker counts in both years of our study (2006: $r^2 = 0.66$, $F_{(1,49)} = 99.3$, $p < 0.001$; 2007: $r^2 = 0.39$, $F_{(1,16)} = 12.0$, $p = 0.003$). Post hoc tests indicated that all groups differed significantly in 2006 and in 2007, with $p \leqslant 0.003$. However, the oldest group in 2007 showed significantly more labeling than the oldest group in 2006 ($p = 0.005$) and was not significantly different from the youngest ($p = 0.098$) and middle-aged ($p = 0.997$) groups in 2006 (Fig. 3). Thus, the data were further evaluated with a two-factorial ANOVA, which indicated an effect of age ($F_{(2,64)} = 36.3$, $p < 0.001$) and year ($F_{(1,64)} = 25.7$, $p < 0.001$) but no interaction between the two ($F_{(1,64)} = 0.1$, $p = 0.716$). In drones, a small but significant overall decline was measured ($r^2 = 0.10$, $F_{(1,53)} = 5.6$, $p = 0.021$) and post hoc tests showed that this reflected lower values in the oldest group compared with the young drones ($p = 0.049$) and middle-aged drones ($p = 0.028$) (Fig. 3).

It proved difficult to obtain reproducible labeling using commercially available apoptosis assays. Despite clear positive controls, the amount of apoptosis could not be reliably quantified in any

experimental group, precluding the comparison between the amount of cell proliferation and cell death. As a consequence, all subsequent studies focused on cell proliferation.

The count of labeled nuclei in one gut section in the *diutinus* winter bees was 143 (95% C.I.: 110–175, $n = 20$) and thus it was significantly lower than in both groups of young workers (Dunnett's T3 post hoc test: $p_{2006} = 0.009$; $p_{2007} = 0.001$) but significantly higher than the old foragers measured in 2006 ($p = 0.002$). The *diutinus* group had most similar counts to the middle-aged workers ($p = 0.767$) and the 2007 group of old workers ($p = 0.589$).

The simultaneous assessment of age and behavioral task in middle-aged workers in a single-cohort hive with respect to intestinal cell proliferation indicated an effect of behavior but not age (Fig. 4). Nursing bees showed a higher amount of cell proliferation than foragers ($F_{(1,41)} = 4.6$, $p = 0.038$) but there was no significant difference between 8 and 33 days of age ($F_{(1,41)} = 0.6$, $p = 0.459$) and no interaction between the two factors ($F_{(1,41)} = 0.0$, $p = 0.974$). Taken together, the counts in this experiment were most similar to the middle-aged workers measured before, the winter workers, and the 2007 group of 41-day-old workers (Fig. 4).

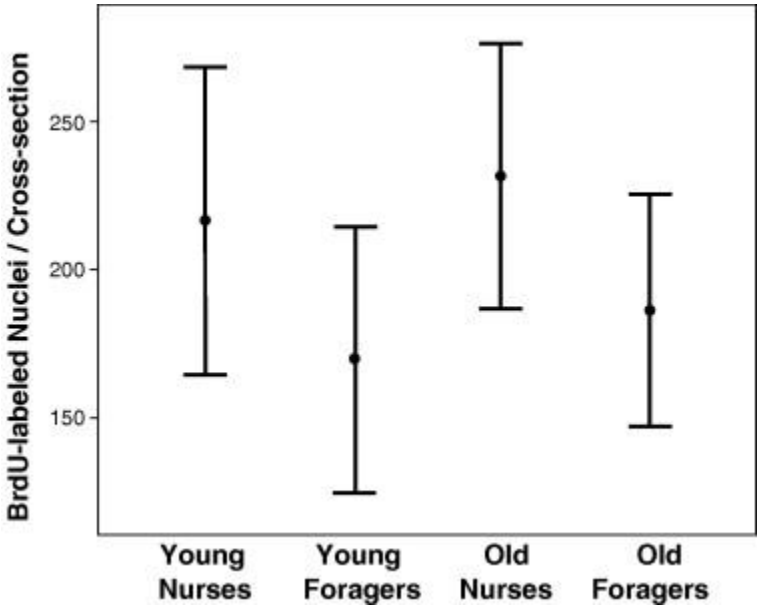

Fig. 4. Among middle-aged workers, behavioral profile but not age influenced the amount of intestinal cell proliferation. Shown are group means and 95% C.I.

In the queenless hive, numerous eggs were produced, but little brood developed during the experimental time period and workers were observed cannibalizing eggs. The queenless colony condition was accompanied by little foraging or other typical worker activity. Although only five marked workers were detected laying eggs, dissected ovaries confirmed a more general worker reproductive activation. In our sample, all ovary activation levels were observed with approximately equal frequency. Intestinal cell proliferation was generally low (107 (81–132), $n = 27$) in the workers from the queenless hive, and it was not significantly correlated with ovary activation level (Spearman's rho = 0.1, $n = 27$, $p = 0.806$).

Experimental feeding with hydroxyurea significantly decreased the life expectancy of workers that moved freely in the colony (Mantel-Cox Log rank test: $\chi^2 = 7.7$, $n = 40$, $p = 0.005$) and of caged worker bees ($\chi^2 = 28.1$, $n = 40$, $p < 0.001$; Fig. 5). The cell proliferation counts of the treated bees were slightly lower than those of the untreated bees but the difference was not significant ($F_{(1,28)} = 3.3$, $p = 0.079$) and counts were generally high compared with the earlier experiments (treated: 253 (209–297), $n = 15$; untreated: 322 (254–389), $n = 15$). The labeling pattern of the treated bees did not reveal any irregularities in localization or morphology of the labeled nuclei.

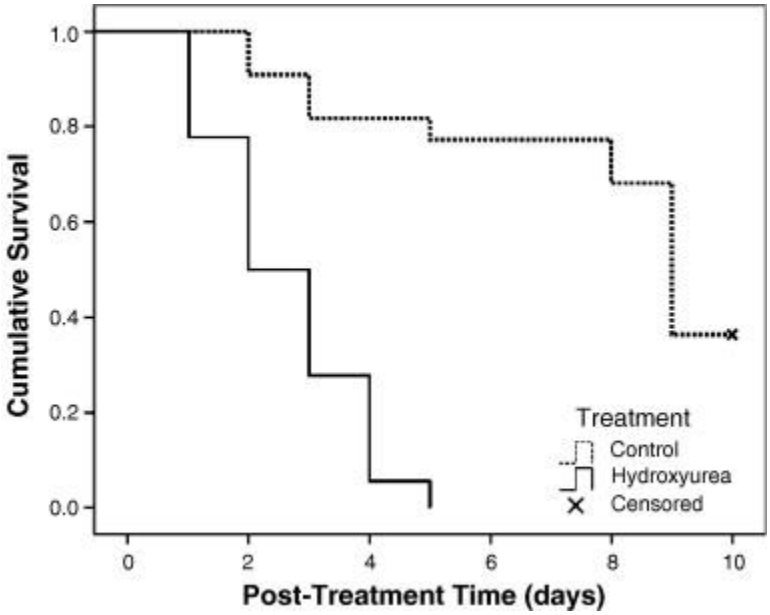

Fig. 5. Hydroxyurea treatment shortened subsequent survival significantly in 8-day-old workers (cumulative survival of 40 workers caged within their hive are shown).

4. Discussion

The honeybee intestine is characterized by relatively high amounts of cell proliferation as indicated by *in vivo* incorporation of the thymidine analogue BrdU. The appearance of the nuclear labeling suggests that these proliferative cells are homologous to the stem and/or progenitor cells in the *Drosophila* midgut, located at the bottom of the intestinal crypts ([Micchelli and Perrimon, 2006] and [Ohlstein and Spradling, 2006]). This interpretation is supported by earlier studies using conventional histological techniques (Snodgrass, 1956). The insect intestine may thus be an important general exception to the concept of adult insects as largely postmitotic organisms (Finch, 1990). No other somatic tissues with consistent mitotic activity were identified in adult honeybees, although sporadic BrdU labeling of pericardial cells was found and other studies have suggested that hemocytes may proliferate in honeybees under special circumstances (Amdam et al., 2005). Feeding of hydroxyurea reduced remaining life expectancy without a significant decrease in intestinal cell proliferation, which could indicate the existence of another population of vital, proliferative cells that were affected by hydroxyurea but were not detected in our studies.

Intestinal stem cell replication may be limited in mice and humans (Lobachevsky and Radford, 2006), which is expected to create some age-related decline in intestinal cell proliferation. In

honeybees, the amount of intestinal cell proliferation showed an age-related decline in queens, drones, and workers over the entire lifespan. The decline was most apparent in queens and workers: Newly emerged individuals showed over twice the amount of cell proliferation than individuals that were about 6 weeks old. It cannot be ruled out that the adult intestine is still growing, by means of cell proliferation, to its full adult size in the first few days after emergence. However, this potential explanation for the initial high counts does not apply to the further decline from the second to the third age class. The second age class represented sexually mature queens and drones, as well as workers towards the end of their in-hive tenure (Winston, 1987). At the older age, queens were still young relative to their lifespan but they were already established egg layers (Winston, 1987). Workers and drones were comparatively old, with typically >80% at these ages dead under normal summer conditions ([Rueppell et al., 2005] and [Rueppell et al., 2007]). Even though cell proliferation counts at this advanced age were comparatively low in all groups, all groups were significantly different from zero and some labeled nuclei were found in all individuals. Queens and *diutinus* workers were considerably longer-lived than 42 days and our data suggest that they are characterized by a constant, low level of intestinal cell replacement. In fact, the oldest queens and the *diutinus* worker group showed slightly more intestinal cell proliferation than the 41/42 day olds. This suggests that the intestinal stem cells have sufficient proliferative capacity to maintain the adult honeybee gut over a relatively long period when cell turnover is low. Our findings do not support the hypothesis that intestinal cell senescence determines organismal aging and death in honeybees.

It is of special note that the long-lived queens exhibited lower initial cell proliferation than the shorter-lived workers, contrary to the prediction that greater somatic resource allocation is associated with longer life. The high amount of cell proliferation in young workers may be driven by a high digestive activity because these young workers serve a prime digestive role in the honeybee society by converting pollen into highly nutritious, processed glandular secretions that are then fed to larvae, other workers, drones, and queens ([Crailsheim et al., 1992] and [Crailsheim, 1998]). Pollen is difficult to digest (Crailsheim, 1990) because the proteinaceous pollen grain interior is protected by a tough, cuticularized pollen wall of complex structure (Roulston and Cane, 2000). Most of the digestive processes occur in the midgut (Keller et al., 2005). Although the midgut epithelium is partially protected by a peritrophic membrane (Richards and Richards, 1977), a higher digestive activity may lead to elevated levels of cell damage because the epithelial cells are metabolically active and protection is imperfect. Brood rearing is believed to be a major factor impacting worker lifespan due to the transfer of protein from the workers to the brood ([Maurizio, 1950] and [Amdam et al., 2004]). The ultimate causes for the longevity cost of brood rearing are not yet resolved but could include an elevated digestion activity. The demand for pollen digestion during nursing may lead to increased damage to the cells of the intestinal epithelium. This would require higher cell replacement rates, which in turn could lead to stem cell exhaustion (Rando, 2006) and intestinal senescence.

Further evidence for the hypothesis that intestinal cell proliferation reflects socially-driven digestive demand more than somatic maintenance at the individual level comes from the decoupling of chronological age and behavioral profile, which showed that nursing is associated with increased cell proliferation compared with foraging, regardless of age. Concomitantly, nurse bees have a higher digestive enzymatic activity than foragers, corresponding to their social role (Grogan and Hunt, 1984). Because social role typically coincides with age (Winston, 1987), the

age-related decline of cell proliferation in workers under normal circumstances could reflect the age-dependent shift from nursing to foraging. Correspondingly, winter workers show little digestive activity (Crailsheim, 1990) and lower proliferation of intestinal cells than other in-hive bees.

Enhanced digestive activity could lead to higher cell turnover in the gut epithelium because the intrinsic cell metabolism is up-regulated for secretion and absorption, or because the epithelial cells incur more extrinsic damage from the digestive processes in the gut lumen. Alternatively, the high proliferative activity may reflect crypt growth and eventual crypt division to provide more epithelial surface area in anticipation of digestive demand (Wilson and Potten, 2004). This would be expected before the demand for the highest digestive activity was experienced. The high amount of cell proliferation in newly emerged workers in spite of little pollen consumption (Hrassnigg and Crailsheim, 2005) is consistent with this interpretation. Digestive demand can also account for lower intestinal cell proliferation in drones because they rely on the preprocessed food jelly of the nurse bees (Hrassnigg and Crailsheim, 2005). However, the digestive demand hypothesis alone cannot account for the high intestinal cell proliferation of young queens, because they are fed by nurse bees with easily digestible proteinaceous secretions in the same manner as older queens (Crailsheim, 1990).

Reproductive workers showed overall low amounts of cell proliferation in their intestines without a significant relation to the degree of ovarian activation. This result suggests that reproductive competition among workers may decrease intestinal cell proliferation, which could result from resource re-allocation from somatic to reproductive functions (Amdam and Omholt, 2002) or from a decreased digestive demand on workers under queenless conditions. The latter of these two explanations is favored by the fact that the degree of ovarian activation was not negatively correlated with the amount of intestinal cell proliferation. The lack of the predicted relationship suggests that there is no direct physiological link between the only two known stem cell compartments (intestine and germarium) in adult honeybees. At the organismal level, the long lifespan of reproductive queens also contradicts a negative trade-off between reproduction and somatic maintenance (Corona et al., 2007). However, the mortality dynamics of reproductive workers have yet to be determined.

As predicted, the experimental feeding of hydroxyurea drastically shortened worker lifespan. However, our treatments did not abolish the activity of the intestinal stem cells. It is difficult to calculate the exact amount of hydroxyurea administered but the ingested quantity was probably in the range of 0.3–3 mg/g, based on the average worker body weight and estimated food consumption. Hydroxyurea has a general cytostatic effect well below these concentrations (Timson, 1975) and the resistance of the honeybee intestinal stem cells therefore seems remarkable. The significant, repeatable treatment effect on survival provides independent confirmation that the dose used was biologically active. The BrdU-incorporation pattern in hydroxyurea-treated workers was similar to that observed in untreated controls, and the number of labeled cells counted was significantly higher than that for several other experimental groups, suggesting that functional cell replacement in the intestinal epithelium was ongoing in the presence of hydroxyurea. The toxicity of hydroxyurea thus could be due to effects on non-replicative cells or may reflect an impact on an essential covert population of proliferating cells (e.g. hemocytes: Amdam et al., 2005).

In conclusion, our study has demonstrated a high replicative activity of stem cells localized at the base of the intestinal crypts in honeybees. This population of stem cells is insensitive to hydroxyurea. The amount of intestinal cell proliferation showed an age-related decline in drones, queens and workers and was influenced by worker behavioral status. These effects are better explained by changing digestive demands and the associated need for cell replacement in the intestinal epithelium (Wilson and Potten, 2004) than by intrinsic senescence of the intestinal stem cells (Ohlstein and Spradling, 2006). Our results did not support the hypothesis that the pronounced aging plasticity in honeybees is driven by different rates of intestinal cell replacement. Further comparative studies, particularly in the context of *in vitro* measures of the replicative potential of intestinal stem cells are needed to evaluate the relevance of this simple but potentially powerful model system for studying of the relationship of stem cell capacity and aging (Rando, 2006).

Acknowledgements
We thank Esther Leise and Dennis LaJeunesse for practical help and technical advice with the cellular assays, and Stephanie Christine, Jackie Metheny, and Preston Gardner for help with the experiments. Greg Hunt generously provided a hive of wintering bees. Comments from two anonymous reviewers helped to improve the manuscript. This research was funded by the American Federation for Aging Research and a National Science Foundation grant (#0615502) to O.R.

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